M·14

PCT/ SE 99 / 0 1 7 4 1

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

Intyg
Certificate

SE 99/1741

4

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

---

(71) *Sökande* *Probi AB, Lund SE*
Applicant (s)

(21) *Patentansökningsnummer* *9900371-7*
Patent application number

(86) *Ingivningsdatum* *1999-02-04*
Date of filing

Stockholm, 1999-12-13

För Patent- och registreringsverket
For the Patent- and Registration Office

Anita Södervall
Anita Södervall

Avgift
Fee

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

PATENT- OCH REGISTRERINGSVERKET
SWEDEN

Postadress/Adress
Box 5055
S-102 42 STOCKHOLM

Telefon/Phone
+46 8 782 25 00
Vx 08-782 25 00

Telex
17978
PATOREG S

Telefax
+46 8 666 02 86
08-666 02 86

NEW USE

The present invention refers to the use of one or more bacterial strains to reduce the level of inflammatory markers such as IL-6 and reactive oxygen species (ROS) in mammals including man.

The present invention refers to the use of a bacterial strain giving rise to increased amounts of propionic acid in the gut for the manufacture of a medicament for reduction of the level of IL-6, the adhesion of monocytes to endothelial cells and the level of reactive oxygen species, ROS, in mammals including man. A high level of ROS is a characteristic of proinflammatory and inflammatory states, as well as high levels of the cytokines interleukin 1 and interleukin 6, and the adhesion of monocytes to endothelial cells.

The bacterial strain is preferably a strain of *Lactobacillus* or *Propionibacterium*.

According to a preferred embodiment of the invention the bacterial strain is a *Lactobacillus plantarum* strain, such as *Lactobacillus plantarum* 299v, deposition number DSM 9843.

The invention also refers to the use of a bacterial strain giving rise to increased amounts of propionic acid in the gut for the manufacture of a medicament for the prophylaxis and/or treatment of chronic inflammatory diseases. Chronic inflammatory or proinflammatory diseases which can be treated according to the invention can be induced by different bacteria, such as Chlamydia pneumoniae and Helicobacter pylori, or toxic substances, such as nicotine.

A preferred use according to the invention is for the prophylaxis and/or treatment of rheumatic diseases.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the reduction of the adhesion of monocytes to endothelial cells after treatment with fermented oatmeal gruel;

Figure 2 shows the reduction of the adhesion of monocytes to stimulated endothelial cells after treatment with fermented

oatmeal gruel;

Figure 3 shows the generation of ROS in monocytes before and after treatment with fermented oatmeal grual.

EXPERIMENTAL

The purpose of this study was to determine the influence of a probiotic bacterial strain on a group of ten healthy volunteers. Each person was given 25 ml/d for a number of weeks of a concentrated oatmeal gruel fermented with *Lactobacillus plantarum* DSM 9843 (containing $1x10^9$ cfu/ml).

Methods

Peripheral mononuclear cell (PBMC)

PBMC were isolated from heparinized blood derived from healthy smoking volunteers by density-gradient centrifugation. The blood was diluted (1:1) in PBS. 25 ml of diluted blood was immediately layered over 15 ml Ficoll-Paque and centrifuged (1900 rpm, 40 min, 22°C). The mixed mononuclear cell band was removed by aspiration and washed with PBS. Isolated PBMC was counted and divided into two parts. One part was used to determine intracellular ROS production, second one was used to adhesion assay. The mononuclear cell preparation consists of approximately 30 % monocytes and 70 % lymphocytes.

Endothelial cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords by collagenase digestion as described by Jaffe. In brief, vein of umbilical cords were perfused with PBS to remove blood cells, filled with 0,1 % collagenase (Ia type) and left for 10 min at 37°C. The endothelial cell (EC) suspension was supplemented with FBS and centrifuged at 1200 rpm for 10 min. EC were cultured in medium 199 under humidified 5 % CO_2 in room air at 37°C. The medium also contained 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 20 mM HEPES, 20 % FBS and 50 µg/ml endothelial cell growth supplement (ECGS). HUVEC were cultured

in gelatin-coated 25 cm^2 flasks. The medium was replaced every 2 days until the cells attained confluence (3-5 days). HUVEC purity was assessed by a "coblestone" morphology typical for quiescent EC and factor VIII staining. Confluent HUVEC were detached by 0.01% tripsin/EDTA antagonized by FBS.

Adhesion assay

For adhesion studies HUVEC of the passage 3 were cultured in gelatin-coated 24-well plates. When confluent monolayers were formed, medium was changed to medium 199 containing antiblotics, 20% FBS and 20 mM HEPES without ECGS 18 hours before experiments. Some cells were pretreated with TNFα (500 u/ml) for 18 hours. Freshly isolated PBMC were resuspended in medium 199 with 20 mM HEPES to a concentration 3 - 9 x 10^5/ml. HUVEC were washed with PBS before addition of PBMC (0,5 ml per well) and coincubated for 30 min. The PBMC suspension was withdrawn. HUVEC were washed with PBS and the wells were fixed with formalin, stained with May-Grunwald and Giemsa solution according to Pappenheim and the number of adherent monocytes were counted in 10 separate areas. Data are expressed as percentage of monocyte added. All experiments were performed in duplicate.

The results are given i Figures 1 and 2.

Reactive oxygen species (ROS) production

The measurement of cell oxidation is based on ROS-mediated conversion of nonfluorescent 2',7'-dichloro-fluorescein (DCFH) (loades into cells as 2',7'-dichloro-fluorescein diacetate) into fluorescent DCF with increased fluorescence emission reflecting enhanced oxidative stress. The study was performed by method previously descriebed. In brief, freshly isolated PBMC were resuspended in PBS followed by incubation with 20 μM DCFH with or without PMA (100 ng/ml) for 30 min in the dark. The fluorescence intensity of fluorophore DCF which is formed by peroxide oxidation of its nonfluorescent precursor was detected with cytopfluorimetric assay (FACScan, Becton Dickinson). During cytometric analysis

monocytes and lymphocytes were gated on the basis FSC and SCC. Results are expressed as mean fluorescence intensity.

The results are given in Figure 3.

Interleukin 6 (IL 6)

The level of IL-6 in blood was measured before and after treatment with the fermented oatmeal gruel.

The results are given i the following Table.

Table

Subject no.	IL-6 pg/ml on 11 Dec. 1998	IL-6 pg/ml on 20 Dec. 1998
1	10	9
2	11	5
3	7	11
4	13	10
5	14	8
6	5	5
7	10	5
8	7	8
9	15	9
10	9	6

CONCLUSION

It is suggested that propionate produced in the large gut by colonic microbial fermentation may have an antiinflammatory effect. It is therefore believed that bacterial strains which give rise to increased amounts of propionic acid in the gut will decrease the proinflammatory state connected with different chronic inflammatory disorders in the body.

CLAIMS

1. Use of a bacterial strain giving rise to increased amounts of propionic acid in the gut for the manufacture of a medicament for reduction of the level of inflammatory markers in mammals including man.

2. Use according to claim 1 for the reduction of IL-6.

3. Use according to claim 1 for the reduction of the adhesion of monocytes to endothelial cells.

4. Use according to any of claims 1 to 3, wherein the bacterial strain is a strain of *Lactobacillus* or *Propionibacterium*.

5. Use according to any of claims 1 to 4 for the manufacture of a medicament for the prophylaxis and/or treatment of chronic inflammatory diseases.

6. Use according to claim 5, for the prophylaxis and/or treatment of rheumatic diseases.

7. Use according to any of claims 1 to 6, wherein the bacterial strain is a *Lactobacillus plantarum* strain.

8. Use according to any of claims 1 to 7 of *Lactobacillus plantarum* 299v, deposition number DSM 9843.

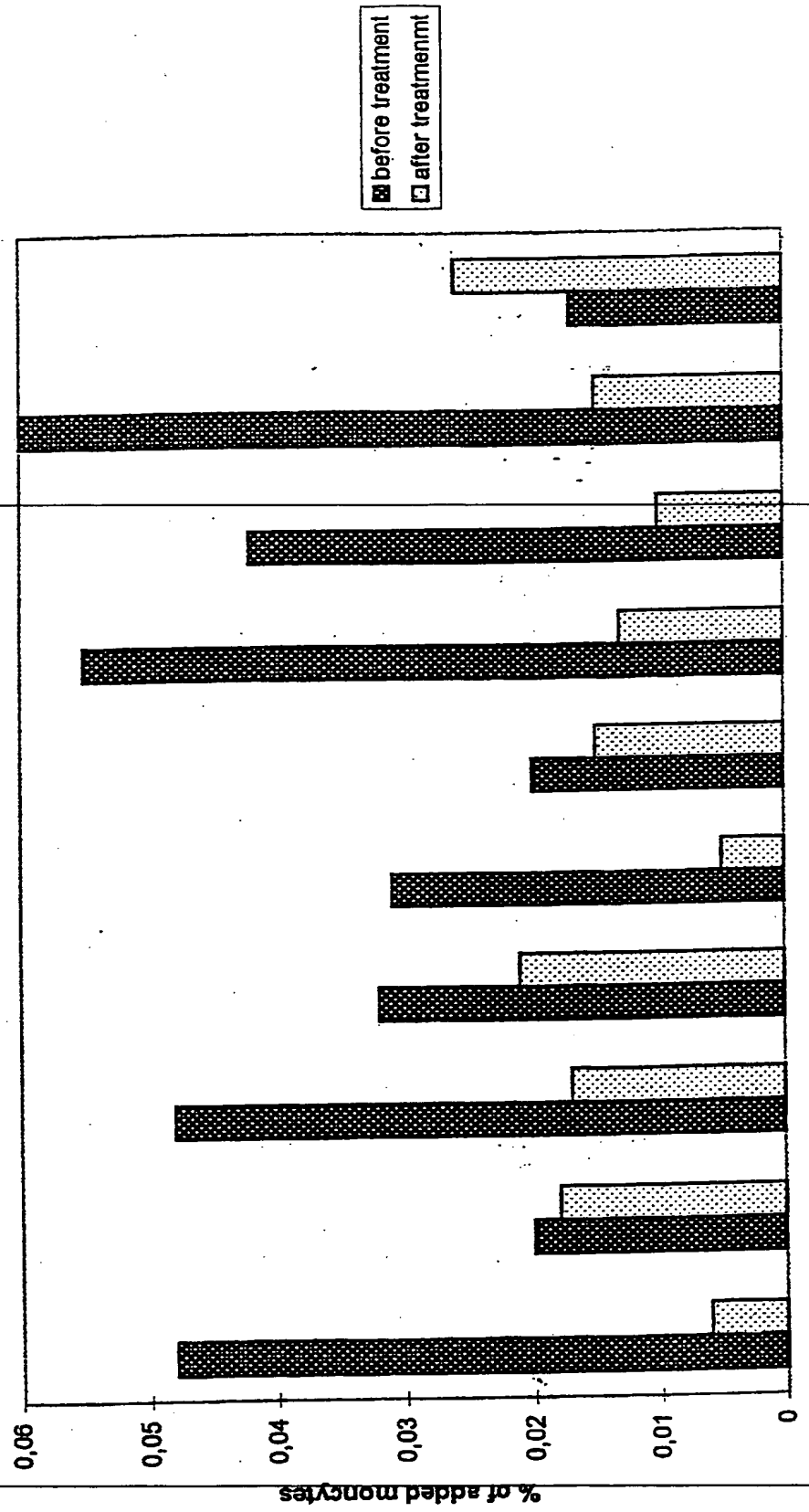

Fig. 1 Adhesion of monocytes to non-stimulated HUVEC before and after treatment

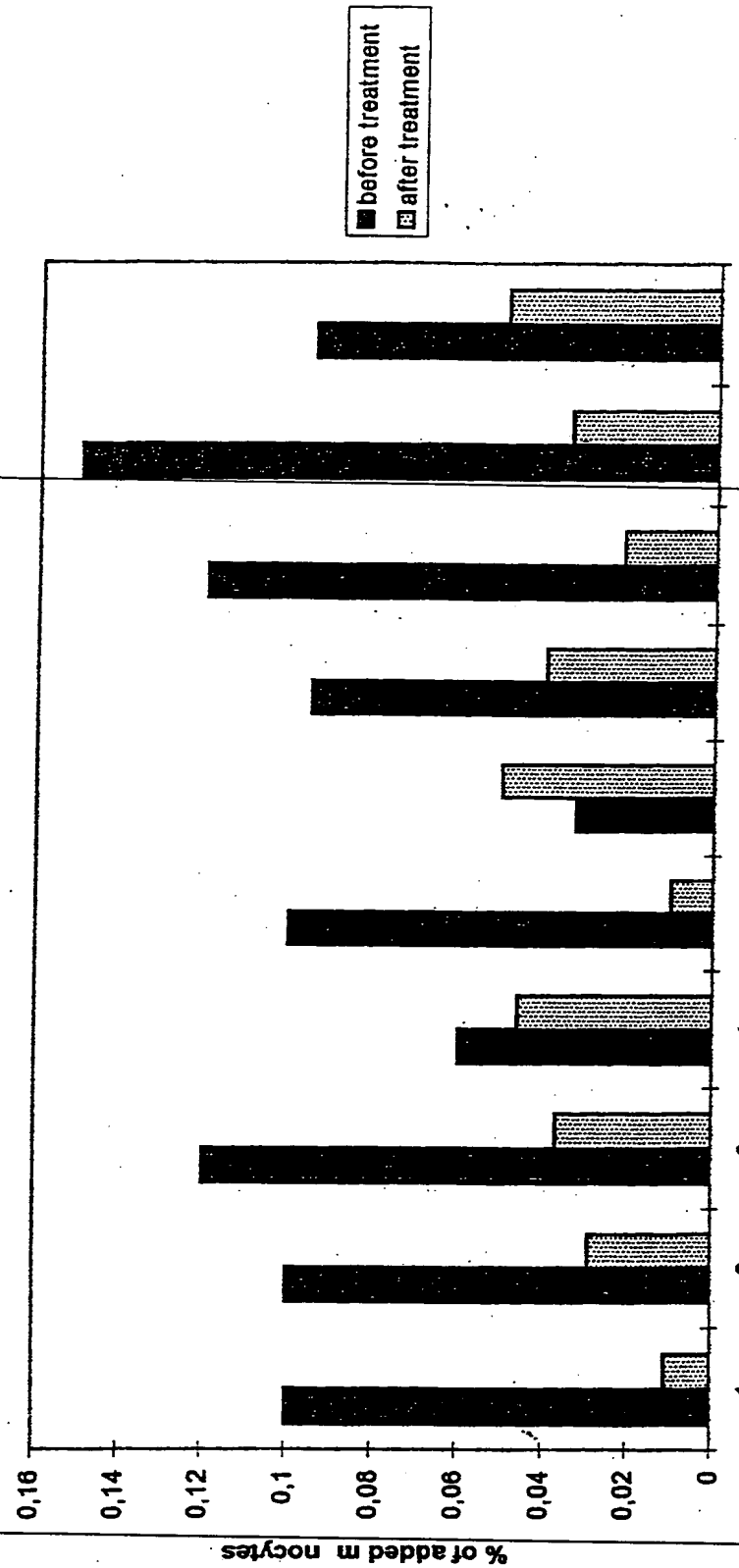

Fig.2 Adhesion of normal resting monocytes to TNF-α-stimulated HUVEC before and after treatment

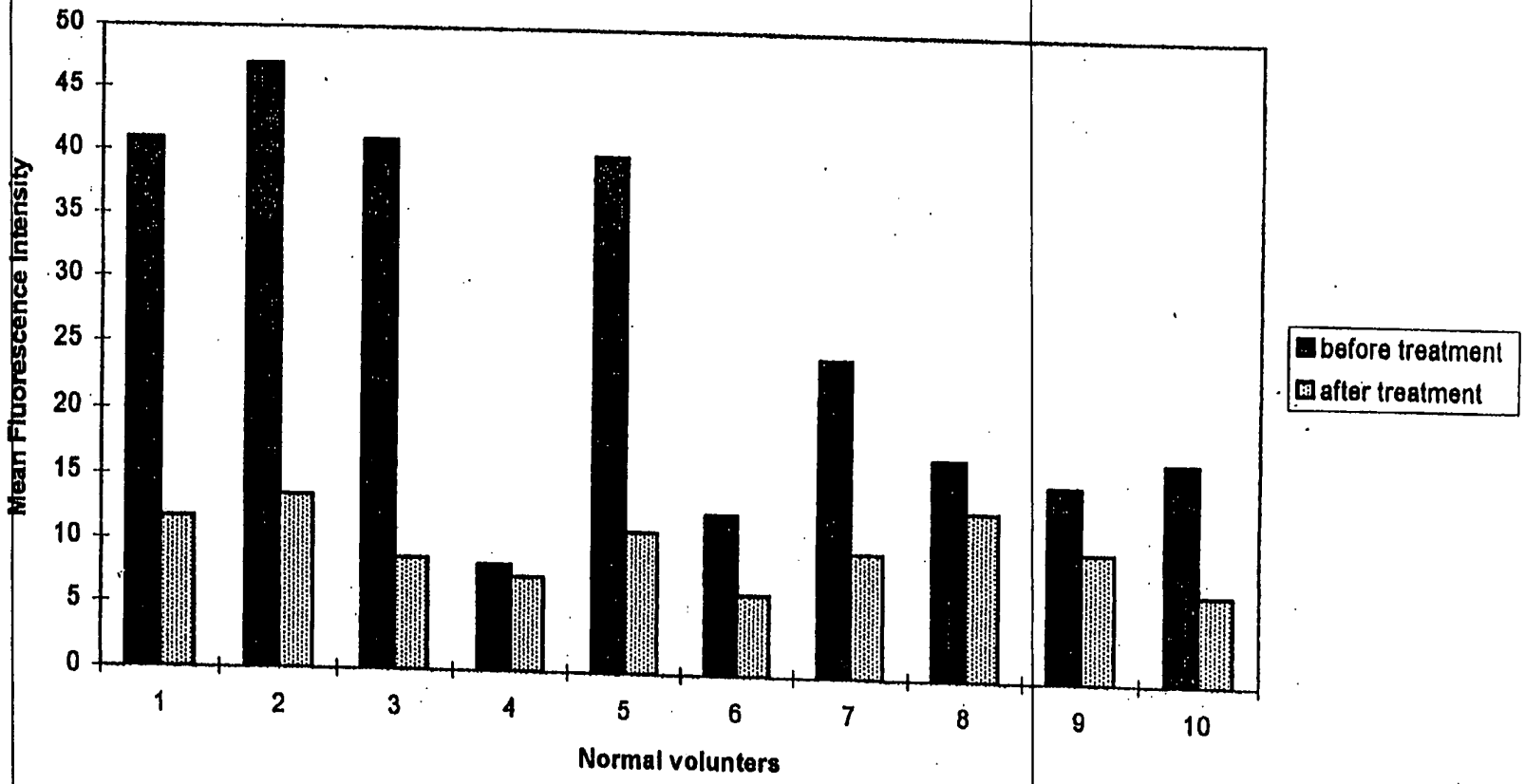

Fig. 3 Generation of ROS in normal resting monocytes before and after treatment